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Differential Upregulation of BDNF mRNA in the Rat Hippocampus after Single and Recurrent Hyperthermia-Induced Seizures

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YALE UNIVERSITY

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Differential Upregulation of BDNF mRNA in the Rat Hippocampus after Single and Recurrent Hyperthermia-Induced Seizures

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Trac M. Duong

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Abstract

Whether febrile seizures produce the neuropathology observed in human TLE patients remains controversial. In this study, we determine the pattern of BDNF mRNA expression in the rat hippocampus after single and recurrent hyperthermia-induced seizures, using the warm-water-immersion method. BDNF mRNA was detected in hippocampal sections by *in situ* hybridization with an ^{35}S-radio-labeled oligonucleotide probe complementary to the sequence of rat BDNF mRNA. Signal from the probe was visualized using plane-film autoradiography, and the intensity was quantified by computerized densitometry. In the hippocampi of both control and hyperthermic rats, BDNF mRNA was most highly localized to the dentate granular cell layer, CA3 pyramidal cell layer, CA1 pyramidal cell layer, the hilus, the molecular layer, CA3 stratum radiatum and CA1 stratum oriens and radiatum, in descending order. The earliest increase in BDNF mRNA expression compared to controls was recorded in the granular cell layer after 3 seizures. By 9 seizures, an upregulation of BDNF mRNA was observed in all 8 measured regions of the hippocampus. The granular cell layer demonstrated the greatest maximum increase in BDNF mRNA (4.2-fold) compared to control levels. In contrast, the CA1 and CA3 pyramidal cell layers exhibited the smallest maximum increase (1.5-fold) of the regions measured in the hippocampus.

In the dentate granular cell layer, a statistically significant increase in BDNF mRNA expression is observed between the following groups: 3 seizures vs. control; 6 seizures vs. control; 9 seizures vs. control; and 9 seizures vs. 3 seizures. A statistically significant increase in BDNF expression was observed in CA1 stratum oriens and the molecular layer of the dentate gyrus after 9 seizures compared to controls. A strong positive correlation was found between the number of seizures experienced and the seizure duration. In addition, linear regression analysis revealed a strong correlation between the severity of the seizure and its duration. These findings suggest that BDNF mRNA upregulation during hyperthermic seizures protects hippocampal neurons from injury. The pattern of BDNF mRNA upregulation in the hippocampus suggests that cell populations which mount an insufficient BDNF response to seizures may be more vulnerable to injury and death during prolonged seizures. In addition to conferring neuroprotective properties, an increase in BDNF mRNA expression may enhance neuronal excitability and lengthen the seizure duration.

Acknowledgements

I wish to thank Dr. Nihal C. de Lanerolle for his expert guidance, untiring motivation, and generous friendship. Without his kindness, formidable knowledge and devotion to finding the answers, this work could not have emerged. I would also like to thank Dr. Michael Brines for his technical assistance and countless hours of patience. Finally, I wish to thank Mrs. Ilona Kovacs for her caring encouragement and vibrant spirit.

Table of Contents

Introduction..1

Statement of Purpose and Hypothesis...22

Methods..23

Results..32

Discussion..38

Bibliography..46

Tables and Figures..55

Appendix.. 61

Introduction

I. Temporal Lobe Epilepsy

Epilepsy is a chronic neurologic disorder that is characterized by spontaneous, recurrent seizures. The term seizure comes from the Latin *sacire*, meaning "to take possession of." A seizure is defined as "a paroxysmal event due to abnormal, excessive, hyper-synchronous discharges from an aggregate of central nervous system neurons. The location and distribution of these abnormal discharges determines the outward manifestations of the seizures. The symptoms can range from involuntary motor activity, such as convulsions, to psychic or experiential symptoms not obvious to an outside observer. A person can experience a single episode of seizures during their lifetime due to an avoidable or correctable cause and not be diagnosed as having epilepsy. In order to have epilepsy, a person must have recurrent, unprovoked seizures that are due to a chronic, underlying cause (1).

The incidence of epilepsy, worldwide, is approximately 0.3 to 0.5%. The prevalence of epilepsy in the United states is 6 to 7 per 1000, and 40 to 50 new cases develop annually per 100,000 (2). By comparison, 5 to 10% of the population will experience at least one seizure in their lifetime (1). In the general population, the risk of epilepsy increases from 1% at birth to 3% by 75 years of age (3). In two-thirds of the cases of epilepsy, the etiology is not identified. However, certain epileptic disorders manifest distinctive clinical and pathological characteristics which suggest a specific underlying etiology and are considered epilepsy syndromes (1). Thus, epilepsy

is not a single disorder but consists of a variety of diseases characterized by chronic, recurrent seizures.

The different epilepsy syndromes are currently classified into separate categories based on clinical history, electroencephalographic findings, and etiology. The localization-related epilepsies and syndromes are characterized by partial seizures, in which seizure activity is limited to a discrete region of the brain. The generalized epilepsies and epilepsy syndromes are characterized by generalized seizures, in which seizure activity originates in both cerebral hemispheres (4).

Partial seizures are the result of focal seizure activity in the brain, and this category is subdivided into simple and complex partial seizures. Complex partial seizures are characterized by focal seizure activity accompanied by an impairment of consciousness, whereby, the patient is unable to respond appropriately to environmental stimuli and has impaired recollection or awareness of the seizure episode. This type of seizure is usually preceded by auras--stereotyped, simple partial seizures which presage the onset of the ictal phase. The beginning of the ictal phase is often marked by a sudden behavioral arrest or motionless stare. From this point onward, the patient becomes amnestic of the seizure. Subsequently, automatisms ensue and can consist of a variety of motor disturbances such as lip smacking, chewing, swallowing, hand gestures ; however, they may be more elaborate behaviors. Typically, the patient is confused following the seizure and recovery may take seconds to hours (1,5).

Nearly one-third of complex partial seizures are not accompanied by EEG changes. Those that do demonstrate focal or lateralized sharp waves, spikes, and slow waves. These waves may follow several different patterns: rhythmic sinusoidal activity or repetitive spike discharges maximal over one region are most common (3). A lack of EEG is associated with brief seizures originating in deep regions such as orbitofrontal cortex or mesial temporal structures.

Temporal lobe epilepsy (TLE) comprises almost two-thirds of all partial epilepsy syndromes (3). Mesial temporal lobe epilepsy (MTLE) is the most common epilepsy syndrome associated with complex partial seizures (1). These seizures arise from the hippocampus or amygdala. Psychic phenomena, visceral sensations, fear, anxiety and olfactory disturbances are commonly reported by TLE patients because their seizures arise in the temporal lobe. The complex partial seizures typically last from 30 to 120 seconds. 50% of patients also report secondarily generalized tonic-clonic seizures. Stress, sleep deprivation, and menstruation are some of the factors which may precipitate the onset of a seizure in this syndrome (3). The correct diagnosis of this syndrome is crucial because of its high incidence among the various epilepsy syndromes and because it tends to be refractory to medical treatment with anticonvulsants in 30 to 40% of patients. However, the intractable seizures of TLE respond extremely well to surgical intervention (1).

II. Anatomy of the Hippocampus

Before discussing the specific pathology found in TLE, a brief description of the hippocampus and its anatomy is necessary. The hippocampal formation lies in the floor of the temporal horn of the lateral ventricle. The individual fields of the hippocampus can be seen as adjacent strips of cortical tissue that run rostrocaudally in the temporal lobe and fold over each other in the shape of the letter "C" (5). The hippocampal formation includes the dentate gyrus (DG), the hippocampus proper also called Ammon's horn, the subicular complex-- divided into the subiculum, prosubiculum, and parasubiculum--and the entorhinal cortex. These anatomic regions are linked by prominent connections that unite them functionally (5). This discussion will focus primarily on defining Ammon's horn and the DG with reference to the other structures when necessary for an understanding of the pathology. A simplified description of neuronal projections will only include predominant ones that have been identified.

Different nomenclatures have been used to describe the hippocampal formation. The system suggested by M. Rose divides the human hippocampal formation into five fields, H1-H5, based on cytoarchitectonic criteria and is often used (6). However, this system is not used widely for description in animal models. Instead, nomenclature proposed by Lorente de No has gained wider acceptance because it can be easily applied to descriptions of the human as well as animal brain (5). Based on studies of Nissl, fiber, and Golgi preparations, this system is notable for dividing the

hippocampus into four fields, CA1-CA4, and it is this terminology that will be referred to below.

The DG consists of three layers. The principal layer consists of granule cells that send dendrites into the overlying molecular layer. Collectively, these two layers are often referred to as the fascia dentata (5). Dentate basket pyramidal cells also occupy the granule cell layer and give rise to GABAergic fibers that surround the granule cell bodies (7). The granule cells send axons, known as mossy fibers, to the proximal dendrites of the CA3 neurons and to the third layer of the DG--the hilus or polymorphic layer (8,9). The hilus contains several types of neurons. One class is the mossy cell that send fibers to the inner molecular layer (10). A second cell type is immunoreactive for peptide somatostatin and GABA. There is evidence to suggest that the cells of the hilus send projections exclusively to the DG (10). Cells of the DG do not project outside of the hippocampal formation (4).

The hippocampus proper is divided into three fields, CA1-CA3. The area that Lorente de No termed CA4 is now included by many authors as the hilus. If the hippocampus is visualized in the shape of the letter "C," field CA3 borders the hilus of the DG where it terminates. CA2 follows the distal portion of CA3. The border between CA3 and CA2 is distinguished by a lack of mossy fiber input from the DG. CA1 lies adjacent to CA2 and is characterized by a more heterogeneous cell population than the other two hippocampal fields. It is estimated that 9.4% of its cells are interneurons and the rest are pyramidal cells (11).

All three fields share a similar laminar arrangement consisting of one cellular layer called the pyramidal cell layer. The border deep to the pyramidal layer, and formed by their axons, is termed the alveus. Between the alveus and the pyramidal cell layer is the stratum oriens--formed mostly by basal dendrites from the pyramidal cells and a population of tangentially oriented interneurons. Superficial to the pyramidal cell layer is the stratum lucidum, a layer exclusive to CA3 that is occupied by mossy fibers of the DG. CA3 is unique from CA1 and CA2 because it is the only hippocampal field to receive mossy fiber innervation from dentate granule cells. The next superficial layer, common to all three fields, is the stratum radiatum where the pyramidal cells of CA2 and CA3 receive associational projections from other rostrocaudal levels of the hippocampus in addition to subcortical inputs. CA1 receives projections from CA2 and CA3 in stratum radiatum and stratum oriens through the Schaeffer collaterals. Next, CA1 pyramidal cells project predominantly to the subiculum (12). The most superficial layer, the stratum lacunosum-moleculare, contains perforant pathway projections from the entorhinal cortex which provide input to hippocampal pyramidal cells in CA1 and CA3.

The subiculum lies adjacent to CA1 and their pyramidal cell layers overlap each other at their borders. Lorente de No applied the term prosubiculum to this overlapping region, and this term is used subsequently by some authors. However, contemporary authors consider most of the prosubiculum as part of the subiculum proper rather than as a distinct region since they are histologically similar (13). Although there is an overlapping region, the border

between CA1 and the subiculum is defined by a cell-free zone between the respective pyramidal cell layers (5). The subiculum is comprised of a pyramidal cell layer with apical dendrites extending superficially to form a wide molecular layer. The deepest portion of the pyramidal cell layer contains smaller neurons analogous to those in the polymorphic layer (14). Some entorhinal fibers of the perforant pathway also terminate in the subiculum (15).

With the exception of the perforant pathway from the entorhinal cortex which reaches all hippocampal fields in parallel, information flow from the DG proceeds through the hippocampus in a serial and unidirectional fashion (5). After receiving input from the entorhinal cortex, the DG relays axons, the mossy fibers, to CA3 and the hilus (8,9). In turn, CA3 pyramidal cells provide input to subcortical regions and to CA1 (9). Finally, CA1 projects to the subiculum (12).

III. Neuropathology of TLE

Temporal lobe epilepsy is strongly associated with a specific neuropathology known as hippocampal sclerosis. First described in 1825, sclerosis refers to the gross appearance of a hard, shrunken hippocampus frequently found in autopsy studies of patients with a history of chronic epilepsy (16). The first microscopic study of this pathology was done by Sommer in 1880, using tissue from an epilepsy patient who manifested clinical symptoms similar to those we now attribute to TLE. In his report, he described a specific pattern of neuron loss in the hippocampus. Particularly, he noted extensive loss of pyramidal neurons in what is now considered CA1

and prosubiculum (17). This region is now referred to as Sommer's sector because the pattern of loss in this region is a consistent finding in TLE. He also observed loss of the granule cells and hilar neurons in the DG (17).

These results were corroborated by Bratz in 1899. In addition to neuron loss in Sommer's sector and the hilus, he documents an area of pyramidal cells in CA2 and CA3 that were resistant to injury . He also found complete preservation of subicular neurons, separated by a sharp boundary from the extensive prosubicular damage. His study drew from 50 autopsy cases of chronic seizures stemming from different causes, including syphilis and cysticercosis. From his investigation, he observed hippocampal sclerosis in 50% of the cases and concluded that not all epilepsy is caused by hippocampal pathology (18).

Many early studies report an association between seizures and hippocampal sclerosis (19). Stauder was one of the first to demonstrate an association between hippocampal sclerosis and temporal lobe seizures, specifically (20). Reviewing autopsy material from patients with chronic seizures, he observed that 92% of autopsy cases with hippocampal sclerosis had signs of complex partial temporal lobe seizures. In contrast, 88% of those epileptics without hippocampal pathology were negative for temporal lobe signs. A similar conclusion can be drawn from the work of Margerison and Corsellis who used EEG criteria to identify patients with temporal lobe seizures. Hippocampal sclerosis was present in 58% of cases exhibiting TLE.

Surgical evidence not only links TLE and hippocampal sclerosis, but also suggests that hippocampal sclerosis may produce or contribute to the chronic seizures that characterize TLE. For example, better seizure control is obtained for intractable TLE patients when surgical treatment includes resection of the hippocampus. A recent study reports a 69% seizure control rate for TLE with total hippocampectomy versus only 38% with partial removal (21). In a separate study, hippocampal sclerosis was the only pathological substrate found in 83% of patients that achieved seizure control after surgical resection (22). Collectively, these findings suggest that hippocampal sclerosis has a functional role in seizure formation.

Modern research has confirmed and elaborated on the initial description of hippocampal pathology given by Bratz and Sommer. Using densitometric techniques, Mathern et al. were able to quantify the proportion of neuron loss in hippocampal sclerosis compared to controls: they report a 50% decrease in granule cells; 55% decrease in the hilus and the portion of CA3 terminating in the hilus; 76% decrease in CA1 and prosubiculum, comprising Sommer's sector; and 41% decrease in CA3 and CA2, considered the resistant region by Bratz (5). Mouritzen Dam performed cell counts of hippocampi obtained from TLE patients and found neuron loss in all hippocampal fields and the DG (23,24). Also, there is evidence for interneuron loss in the hilus that is limited to three cell types: somatostatin, neuropeptide Y, and substance P neurons (25,26).

Further findings report hippocampal sclerosis tends to be unilateral in chronic TLE; however, there is bilateral hippocampal

neuron loss. Meenke and Veith found unilateral hippocampal sclerosis in 30.5% of their patient population, but bilateral damage was reported in 56% (27). In a more limited study, the hippocampus contralateral to the side of sclerosis had an average of 30% neuron loss; however, the damage to this side was diffuse and greatest in CA2 instead of Sommer's sector (28).

Research indicates that TLE pathology is not limited to the hippocampus, but may extend to other regions of the brain. In addition to neuron loss in Ammon's horn, concomitant damage has been found in the amygdala and temporal neocortex of some patients with TLE. Falconer et al. coined the term mesial temporal sclerosis (MTS) to describe the pattern of hippocampal sclerosis coupled with additional injury outside of the hippocampus (29). According to one study, damage to the amygdala was seen in 64% of patients with a finding of hippocampal sclerosis (30). Studies of surgical specimens estimated that neuron loss in the amygdala was 60% of controls (31). Recent MRI studies suggest involvement of both the amygdala and hippocampus in MTS (32). In fact, reviews of the literature revealed that MTS is the predominant pathology in temporal lobe resections for intractable TLE (39). Frequency of MTS is reported from 49% to as high as 76% (29,40).

In addition to neuronal injury and loss, there is growth of new fibers in a proportion of TLE cases--a phenomenon called sprouting (33). The best documented sprouting is in granule cell mossy fibers which send novel, recurrent collaterals into the inner molecular layer of the DG. This phenomenon occurs predominantly in mesial temporal lobe epilepsy (MTLE)--TLE not stemming from an

epileptogenic mass lesion (25). A small amount of mossy fiber sprouting into the inner molecular layer is detected in TLE patients with temporal mass lesions (MaTLE); however, densitometry reveals that sprouting is significantly greater in MTLE. (34). In addition to mossy fibers, three other types of axons have demonstrated sprouting in MTLE. NPY axons are increased in density in the molecular layer of the DG (35). Likewise, somatostatin fibers are increased throughout the molecular layer of the DG, preferentially synapsing with apical dendrites of granule cells (25). Sprouting is also observed in fibers immunoreactive for substance P in the granule cell layer of the DG (36).

IV. Etiology of TLE--A Possible Role for Febrile Convulsions

A number of different etiologies have been proposed as causes of TLE. These factors are derived mostly from histologic examination of lesions found in patients who underwent surgical resection as treatment for intractable TLE (37). One group of lesions is attributed to abnormalities during development and includes findings such as arachnoid cysts, epidermoid cysts and heterotopic grey matter (37). More recently, neuronal migration disorders (NMD) were the most common neuropathological finding in a study of children with medically refractory epilepsy treated surgically (62). Another possible factor is head trauma, suggested by a finding of focal scars and gliosis in the temporal cortex or white matter and supported by a clinical history (29). Vascular malformations and various neoplasms, such as astrocytoma or oligodendrogliomas, have been removed from the temporal lobe

with subsequent control of seizures (38). Inflammatory damage has also been suggested as an etiology based on microscopic evidence of prior necrotizing encephalitis or meningitis. Nonetheless, MTS remains the most frequently reported lesion in TLE patients (39).

In looking for possible etiologic factors of epilepsy, Falconer observed that in the subgroup of TLE patients with a finding of MTS, 60% had a previous history of febrile convulsions (FC) as children (29). Subsequent studies of temporal lobectomy for intractable TLE confirm this finding. In the population of TLE patients with MTS, the percentage with a prior history of FCs is reported between 18-79%, depending on the study (40,41).

Febrile convulsions or seizures are defined as "an event in infancy or childhood, usually occurring between 3 months and 5 years of age, which is associated with fever but without evidence of intracranial infection or defined cause. Seizures with fever in children who have suffered a previous non-febrile seizure are excluded" (42,43). The diagnosis is clinical, and EEG may serve to confirm seizure activity. Although there is not a consensus on the body temperature required to produce an FC, many agree that a temperature of at least 38.5 ^{0}C is required (44). A study by Aicardi found that three-quarters of children with FCs have temperatures of 39 ^{0}C or higher, and one-quarter have temperatures greater than 40 ^{0}C (45). Some hypothesize that it is the rate of rise in body temperature that determines if FCs will occur, but there is no clinical data to support this (46,47).

FCs are the most common form of childhood seizures (48). It is estimated to affect 3 percent of all children, but this rate is higher

in certain populations, reportedly reaching 15% in Guam (49). One-third of children will have a recurrent febrile seizure after their first episode (50). Some studies find that children with FCs are at increased risk for subsequent, unprovoked afebrile seizures, including epilepsy (48). The risk for developing epilepsy after FCs varies by study. Millichap, in a review of the literature, found the rate was 20%--the lowest incidence was 2.6% and the highest was 100% (52). In particular, children with FCs exhibiting complex features--prolonged, focal or recurrent seizures--appear to be at significantly higher risk for subsequent, unprovoked partial seizures. The risk is cumulative with the addition of each complex feature; children with FCs with all three complex features have a 49 % risk of later seizures (51). More recent prospective studies estimate the incidence of epilepsy from 1.3% to 12% (50,53). However, only children with complicated or prolonged FCs have a higher risk for epilepsy (51,54).

The precise nature of the relationship between FCs and the development of MTS and TLE remains controversial (39). Evidence to support the hypothesis that FCs cause neuronal injury--HS and MTS--that results in TLE comes from retrospective studies of patients who underwent surgical resection for treatment of intractable TLE. As previously mentioned, a significant proportion of patients with TLE and MTS have had prior histories of FCs. Additional support for this hypothesis can be found in a study, by Sagar and Oxbury, that documents a significant correlation between prolonged, early childhood convulsions and the severity of hippocampal neuron loss in TLE (55). This re-emphasizes the

prolonged nature of FCs as necessary for producing neuron loss and contributing to an increased risk of recurrent seizures.

More recent data corroborates these findings. Mathern et al. reviewed TLE surgical resection cases and examined the clinical histories for events that involved loss of consciousness greater than 30 minutes or alterations of cognition for more than 4 hours, termed initial precipitating injuries (IPI). 41% of IPIs involved either prolonged seizures or status epilepticus. Patients with IPIs showed more severe neuron loss in the pattern of hippocampal sclerosis than non-IPI patients or age-comparable autopsy specimens (56). Furthermore, patients with IPIs and hippocampal sclerosis had the best seizure control following surgical resection (57). MRI studies have also shown a correlation between prolonged FCs in childhood and more severe forms of MTS (58). Despite these results, prospective and population-based studies of children with FCs have failed to find an association with temporal lobe epilepsy (51,59)

This failure led others to propose that FCs do not cause MTS or TLE, but are merely an age-specific marker for TLE (59). According to this hypothesis, FCs, MTS and TLE are all products of an underlying pathology (39). In one study, MRI was used to examine the brains of members from two families with a marked history of FCs. A similar hippocampal malformation was found in all subjects with FCs or TLE as well as a few unaffected relatives (60). This finding suggests that pre-existing pathology, such as an hippocampal malformation, may predispose children to the development of FCs. In turn, FCs cause MTS that eventually leads to TLE. Another conclusion that can be drawn is that FCs and MTS are independent

phenomena, and they are both caused by the same pre-existing pathology. Animal experiments support this latter scenario. For example, when neuronal migration disorders (NMD) were induced in the brain, NMD rats had lower thresholds for seizures and hippocampal damage than normal controls after exposure to hyperthermia (61). In these NMD rats, hippocampal damage developed independently of seizure activity.

Despite the amount of research published on the subject, the precise relationship between FCs, MTS and the development of TLE remains to be defined.

V. The Hyperthermia Model of Febrile Seizures

In an attempt to resolve the conflicting reports in human studies regarding the role of FCs in TLE, some researchers have turned to animal models in order to gain insight into febrile seizures. One such model, developed by Baram and associates, uses a stream of warm air (42-43 ^{0}C) to elevate the core body temperature of immature rats in order to simulate the febrile state in humans and elicit seizures. The advantages of using an animal model are that pre-existing lesions can be excluded, and the investigator can control both the duration and magnitude of hyperthermia. Thus, hyperthermic seizures can be closely regulated. Stereotyped, reproducible seizures are elicited in 98% of the rats in this model (63). Applying patch clamp techniques to examine this model, Baram was able to show that febrile seizures cause a selective presynaptic increase in inhibitory synaptic transmission that persists into adulthood. In vivo, the

hyperthermic-seizure rats demonstrated enhanced susceptibility to limbic seizures: a subthreshold dose of intraperitoneally-administered kainate produced status epilepticus but only resulted in behavioral hyperactivity in their normothermic controls (64). This finding was substantiated by in vitro data, gathered from experimental rats, that describes self-sustaining excitability recorded in hippocampal-entorhinal cortical brain slices after electrical stimulation. However, subsequent spontaneous seizures were not produced. Normothermic rats and rats exposed to hyperthermia without seizures did not display any changes in hippocampal excitability (64). Baram also reports transient alterations in neuronal structure, but not neuron loss, in the amygdala and pyramidal cell layer of fields CA1 and CA3 brought about by hyperthermic seizures in the rat (65). These elegant experiments demonstrate that febrile seizures can produce long-term changes in hippocampal excitability and susceptibility to subsequent seizures.

In order to examine the issue of whether febrile seizures can produce the hippocampal neuropathology seen in TLE patients, Jiang et al. employed another variation of the hyperthermia model in immature rats (66). Instead of warm air, a temperature-controlled water bath was used to increase the core temperature of the rats. The principal advantage of this warm-water-immersion model, first developed by Klauenberg and Sparber, is a more effective rate of heat transfer, simulating the rapid core temperature elevation in human febrile seizures (48,67). Some authors have speculated that it is the rate of rise rather than an absolute temperature which

triggers a FC (46,47). This model allows for repeated induction of seizures. Some rats with a history of multiple, short-duration FCs develop prolonged seizures upon subsequent exposure hyperthermia. These prolonged seizures are similar to status epilepticus in humans.

The warm-water-immersion model bears several striking similarities to human FCs and TLE. Holtzman reports that the increase in seizure threshold coupled with changes in EEG patterns in maturing rat pups, as they are warmed, resembles the seizure characteristics associated with FCs in young children (68). Additionally, the neuropathology in rats that experienced prolonged seizures displays marked resemblance to pathology observed in tissue resected from human TLE patients: 1) neurodegeneration was confined to the hippocampus, temporal lobe (amygdala, pyriform cortex, entorhinal cortex), and dorsomedial nucleus of the thalamus, 2) minimal degeneration was detected in neocortex, 3) degeneration was seen in the hilus, CA3, and CA1, and 4) the dentate granule cell layer and CA2 were more resistant to injury (66). In contrast, rats that only underwent short-duration seizures did not manifest any detectable signs of neurodegeneration. This finding, in an animal model, corroborates the strong association, initially described by Sagar and Oxbury, between prolonged FCs and the severity of hippocampal neuron loss in human TLE (55). Sprouting of recurrent collaterals from the mossy fibers into the inner molecular layer of the DG was also seen in rats that experienced short-duration, hyperthermic seizures. Furthermore, the degree of

sprouting correlated with the number of short-duration seizures that were induced (66).

Pathology in this model does have some differences from human TLE. One notable departure from human studies is that collateral sprouting was not correlated with the degree of granule cell loss in the rat (66). Another difference is that the pattern of hippocampal neuron loss was similar, bilaterally; whereas, in human studies, there is an asymmetric pattern of hippocampal neuron loss (27, 66). Additionally, it is not known whether spontaneous seizures arise in this model, subsequent to the status epilepticus seizures. However, spontaneous seizures have been documented in all other rat models following treatments that produced status epilepticus--pilocarpine model, kainic acid model, and continuous-stimulation model (69-71). Efforts are currently underway to monitor for this phenomenon.

Although there are differences between the hyperthermia model in rats and human FCs and TLE, the similarities in seizure semiology and neuropathology make it an invaluable model for future study.

VI. The Role of Brain-derived Neurotrophic Factor in TLE

One promising area of investigation examines the potential role of brain-derived neurotrophic factor (BDNF) in epileptogenesis and synaptic modulation. BDNF is a member of the neurotrophin family of trophic molecules which includes nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). BDNF mediates its effects through high-affinity interactions with the

tyrosine kinase receptor, TrkB. Neurons expressing the BDNF gene also contain mRNA for TrkB, indicating that BDNF may function locally via autocrine and paracrine mechanisms (74). During normal development of the PNS and CNS, BDNF promotes survival of cranial and spinal sensory neurons; however, its function in the mature CNS is poorly understood (75,76). BDNF is widely distributed throughout the CNS, but the highest level of BDNF mRNA expression is in the dentate gyrus (DG), CA3 and CA2 pyramidal neurons of the hippocampus (77).

The discovery that seizures can induce an increase in BDNF mRNA expression in cortical and hippocampal neurons led scientists to speculate that its upregulation could be responsible for the sprouting and synaptic reorganization seen in human epilepsy (78). Indirect evidence to support this hypothesis comes from *in vitro* experiments in which exogenous BDNF was shown to protect hippocampal neurons and enhance cell survival against various insults such as hypoglycemia and excitotoxic damage from glutamate (79). In these experiments, excitotoxic injury is mediated by a large influx of Ca^{2+} into the cell. BDNF protects these cells by stabilizing the intracellular $[Ca^{2+}]$ homeostasis; one way it might do this is by stimulating expression of calcium-binding protein calbindin (80,81). There is evidence to suggest that BDNF may directly induce mossy fiber sprouting: in vitro, exogenously administered BDNF has been shown to increase axonal branching of dentate granule cells of the hippocampus (82). From this body of research, it is plausible that BDNF, when induced by brain insults such as FCs, can promote the survival of a certain subpopulation of

hyperexcitable neurons in the hippocampus leading to the formation of an epileptic focus. A second possibility is that an increase in BDNF from basal levels promotes the formation of aberrant synaptic connections through axonal sprouting which results in the formation of abnormal, hyperexcitable circuits. Hypothetically, epileptogenic remodeling following FCs could involve a combination of these processes.

An alternative conclusion is that an increase in BDNF confers resistance to neuronal injury in the hippocampus during simple or short-duration FCs. However, if there is an insufficient BDNF response during prolonged seizure activity such as status epilepticus or complicated FCs, this could result in neuron loss and TLE. This could explain why neurodegeneration is only observed in hyperthermic rats following status epilepticus and not after short-duration seizures alone (66).

Recent research demonstrates that BDNF contributes to epileptogenesis by directly enhancing excitatory synaptic transmission both in vitro and in vivo. BDNF enhanced excitability at mossy fiber synapses when administered to brain slices taken from pilocarpine-treated rats with spontaneous seizures (83). In mutant mice with genetically reduced levels of BDNF expression, seizure induction by kindling was suppressed (84). Conversely, transgenic mice which overexpress BDNF have spontaneous seizures and are more sensitive to seizure induction by kainic acid (85). When TrkB receptors known to mediate the actions of BDNF were blocked in vivo, seizure-induction was inhibited in the kindling rat model of epilepsy (86). The significance of these findings for

human TLE is made more compelling by the report that patients with TLE show an increase in BDNF expression, particularly in the DG, compared to normal human subjects (87,88). Furthermore, BDNF enhances fast excitatory synaptic transmission in the DG when exogenously administered to brain slices taken from human TLE patients (89).

Statement of Purpose

The purpose of this experiment is to determine the pattern and quantity of BDNF mRNA expression in the hippocampus of the rat after single and repeated hyperthermia-induced seizures. These findings will be compared to the pattern of BDNF mRNA expression observed in rats with status epilepticus and non-seizure controls.

Hypothesis

Given the known neuroprotective properties of BDNF, we hypothesize that BDNF mRNA expression is increased in the hippocampus of rats experiencing short-duration, hyperthermic seizures without detectable signs of neuronal injury. In contrast, we predict that an insufficient BDNF mRNA response accounts for the pattern of excitotoxic injury and cell death observed in rats with prolonged seizure duration--such as status epilepticus--and neuronal loss.

Methods

Experimental Design

This experiment was designed to investigate the levels of BDNF expression in the hippocampus of the rat brain as a function of the number of seizures induced by hyperthermia. For this purpose, a model of febrile seizures in the immature rat was used in which seizures could be elicited reliably and repeatedly via exposure of the animal to hyperthermia in a heated water bath. The animals were sacrificed when the correct number of seizures was obtained and their brains were immediately harvested for study. BDNF expression was detected in thin coronal sections of the hippocampus via in situ hybridization with an ^{35}S-labelled 50 mer oligonucleotide probe designed for specific binding to rat BDNF mRNA. The signal from the probe was detected and visualized by autoradiography onto plain film. This technique also allowed for determining the localization of the probe to different regions of the hippocampus. BDNF mRNA expression was measured as the strength of the signal from the probe. This signal was quantified by densitometric analysis of the autoradiogram coexposed with ^{14}C standards.

This study was comprised of four experimental groups only differing in the number of seizures induced by hyperthermia (1, 3, 6 and 9 seizure groups, respectively) plus one control group. Each of the four experimental groups consisted of 3 young Sprague-Dawley rats. For each of the four experimental groups there was a separate, age and sex-matched rat serving as a control, which underwent the exact laboratory conditions and induction trials as

the respective experimental group, with the exception of hyperthermia and seizure activity. Subsequently, these four control rats were assembled as one control group. The rationale for this was that the amount of BDNF mRNA detected by in situ hybridization was similar among these normothermic control rats.

Originally, this study was designed with a larger sample size (n=6), but this number was reduced (n=3) because some subjects died before the correct number of seizures could be induced. Due to the long period of time required to prepare this rat model and perform in situ hybridization, more subjects could not be generated to meet the deadline for this study.

Subjects

The subjects selected for this experiment were male Sprague-Dawley rats obtained at age 15 days. They were housed with their mothers under standard laboratory conditions until they were weaned at ~21 days. By age 22 days, the rats had a mean weight of ~100 g. They were then moved and housed in groups of four with ad libitum access to food and water. A daylight cycle of 12:12 h was maintained. Both control and experimental groups started with 22-day-old rats which grew older as they continued to be stimulated to the correct number of seizures. Although an age comparison between the rat and human brain remains an approximation, it has been suggested that the 5 to 7-day-old rat brain is equivalent to that of a full-term newborn infant. The 15-day-old rat brain corresponds to a human brain that is a few months to 1-year-old, and the 28-day-old rat brain is approximate to the brain of a 2-year-

old child. The febrile seizures associated with the development of TLE in humans occur in the period between 6 months to 5 years of age. Hence, the 22-day-old rat most closely models this period of development in the human brain.

Seizure Induction

All protocols involving animal use were approved by the Yale Animal Care and Use committee. Seizure induction trials were begun on postnatal day 22 by exposing the subjects to hyperthermia. In brief, the subject's core body temperature was raised by immersion in a temperature-controlled water bath maintained at 45^0 C for five minutes or less if seizure activity was elicited. A water temperature of 45^0 C does not produce skin damage at exposures <1 hr. In human subjects, this temperature produces only a mild "pricking pain" which resolves in a few seconds. These trials were conducted using a glass chromatography tank (30 x 30 x 60 cm) containing water to a depth that the animal could stand upright supported by the side of the tank with only its head and forelimbs above water level. At the first sign of seizure onset, the animal was immediately removed from the water and placed in an observation chamber. Seizure latency was measured as the interval between when the rat was placed in the water until its first sign of seizure activity. The seizure duration was measured as the time from seizure onset to when the rat first righted itself and appeared conscious. The state of consciousness was determined by the responsiveness of the rat to one of the following stimuli: tapping on the cage, responsiveness to touch or movement of an

object before its eyes. At the end of this period of observation, the rat was gently dried with a towel and returned to its home cage. This procedure was repeated with an interval of two days between trials until the correct number of seizures was obtained.

Seizure severity was rated based on the appearance of the following seizure characteristics: 0 for no seizure; 1 for facial clonus; 2 for head nodding; 3 for forelimb clonus; 4 for rearing (standing posture aided by tail and laterally spread hind limbs showing increased tone); and 5 for rearing and falling.

Sex-matched control rats underwent the same conditions of induction as outlined above for their experimental counterparts with the exception that they were placed in water maintained at 37^0C for four minutes and did not exhibit seizure activity. Likewise, these trials were begun on postnatal day 22. Control subjects underwent the same number of trials as their respective experimental group.

Tissue Collection

One hour after seizure induction, the rats were anesthetized by inhalation of CO_2 gas for thirty seconds and sacrificed by decapitation. The brains were harvested within three minutes of death and embedded in OCT before being transferred onto dry ice to solidify. The coronal block was stored at -70^0 C.

Cryostat Sectioning

16 um coronal sections were made with a cryostat at -14^0 C. Sections were collected with pre-warmed, amino-alkylated glass slides and stored in cryoboxes at -70^0 C.

Synthesis of BDNF Oligoprobe

To detect rat BDNF mRNA, a 50 mer oligonucleotide complementary to rat BDNF corresponding to nucleotides 746-795 of pig BDNF was used (Leibrock et al., 1989). The sequence of the rat BDNF probe is as follows: 5'-AGTTCCAGTGCCTTTTGTCTATGC-CCCTGCAGCCTTCCTTCGTGTAACCC-3'. This sequence has a GC content of 56%. Hybrids with a higher frequency of G-C pairs are more stable than those with a predominant A-C pair content although a GC content of >65% causes non-specific binding since the thermal stability of the probes is greater (Current techniques). Synthesis was performed by the Yale Oligonucleotide Laboratory with a DNA synthesizer.

Terminal Transferase Labeling Reaction

Oligoprobes for BDNF were labeled with ^{35}S at the 3' end using a kit by Boehringer-Mannheim according to the manufacturer's protocol. The probe was purified by applying 100uL of reaction solution to a Sephadex G-25 spin column that was centrifuged for four minutes at 2,700 rpm. The eluate was collected and activity was determined by liquid scintillation counting (~100,000 cpm). 2uL of 1M DTT was added to the eluate. The probe was stored at -20^o C.

Fixing Sections

In order to maintain morphological integrity of the tissue, retain target nucleic acid intact and allow good accessibility to probes, a paraformaldehyde fixative was chosen for its known cross-linking properties in the following protocol. Slides were transferred directly from a -70^0 C freezer to staining racks and placed in the fixative. Fixation consisted of five minutes in ice cold 4% depolymerized paraformaldehyde (PFA) solution; five minutes in 1X PBS; five minutes in 70% ethanol; and five minutes in 95% ethanol. The slides were allowed to air dry in the staining racks for thirty minutes while covered with Kimwipes to prevent contamination by any particulate matter that may be in the air. All steps were performed at room temperature (RT) except where indicated; all solutions were made with DEPC-water; glassware was rendered RNase-free prior to use.

In Situ Hybridization

Hybridization buffer was prepared fresh assuming 100ul/slide (4X SSC, 20% dextran sulfate, 0.25mg/ml yeast tRNA, 0.25mg/ml poly A, 0.25 mg/ml Salmon testes DNA, 2.5X Denhart's solution, and 70% deionized formamide). The buffer was mixed by vortexing and centrifuged to remove bubbles before being warmed to 42^0 C in a water bath. 2uL of oligonucleotide probe (~100,000 cpm) was added per 100ul of hybridization buffer, and the mixture was vortexed. The fixed slides were placed in a hybridization chamber, and 100ul of hybridization buffer was applied to each slide. To

limit evaporation of this buffer and enhance its distribution over the tissue samples, Parafilm strips were placed over the slides instead of glass coverslips. Air bubbles are more easily eliminated using Parafilm and this promotes even distribution of hybridization buffer over the tissue sample. Humidity inside the chamber was maintained by adding a small amount of solution composed of 4X SSC and 50% formamide to the floor of the chamber before sealing the lid and seams with tape. Incubation proceeded overnight at a temperature of 42^0 C. Oligoprobes hybridize optimally at 5^0 C below T_m-- the temperature of dissociation and reassociation of 50% of the hybrids. Here, it was calculated as $T_m=2^0$ C (A+T bp) + 4^0 C (G+C bp) - 5^0 C.

Post-hybridization Wash and Autoradiography

Coverslips were gently removed and the slides were washed in 2x SSC followed by a one minute wash in 1X SSC, both at RT. The slides were soaked in 1X SSC with 10mM DTT at 60^0 C for thirty minutes before being rinsed briefly in 1X SSC, 0.1X SSC, 70% ethanol, and 95% ethanol. The slides were air-dried for thirty minutes and coexposed to Biomax film with a slide of ^{14}C standards for four weeks at RT.

Analysis of Autoradiograms by Densitometry

The density of grains on the autoradiograms was measured using a microcomputer imaging device (Imaging Software). From this density measurement, the amount of radioactivity could be extrapolated by MCID/M4 software (Imaging Software) from a range

of standard values generated by measuring the densities of ^{14}C standards of known activity coexposed with the hybridized slides at the time of autoradiography. From each rat, autoradiograms of three brain sections were selected for analysis--one representative of the caudal hippocampus, one representative of the middle hippocampus and one representative of the rostral hippocampus. The density of the following regions was measured--in CA1, stratum oriens, the pyramidal cell layer, and stratum radiatum; in CA3, the pyramidal cell layer and stratum radiatum; in the dentate gyrus, the granule cell layer, the molecular cell layer and the hilus (Figure 1). Measurements were obtained for each section, bilaterally. This yielded 16 measurements per brain section.

Statistical Analysis of Data

For each of the eight regions of the hippocampus and dentate gyrus analyzed by densitometry, a mean value was calculated from the rostral, middle, and caudal measurements. Hence, each animal had a calculated mean value for each of the eight hippocampal regions (Appendix I).

Because of the small sample size, a Kruskal-Wallis one-way analysis of variance was performed, on the mean values, to detect overall variance between groups for the eight hippocampal regions examined. Differences in BDNF mRNA levels, between seizure groups and between a seizure group and the control group, were compared using the Mann-Whitney one-tailed U-test.

Statistical linear regression was performed to determine the strength of correlation between seizure grade and seizure duration.

Linear regression was also performed to determine the strength of correlation between the seizure number and seizure duration.

Note: *All of the above procedures were performed by the author except where noted in the text.*

Results

Description of Seizures

The rats initially swam upon being placed in the water. Within a few seconds, they stood up on the floor of the tank supported by their hind limbs. The forelimbs were used for support in this position against the sides of the tank. Some moved along the sides of the tank in this position before becoming stationary for the duration of the 4 min. trial period. Sometimes, seizures began before the entire 4 min. elapsed. The rats were promptly removed from the tank at the first signs of seizure, and placed in the observation cage. Rats that did not experience seizures in the water would lay motionless on their belly until seizures began. The latency period to seizure was 2 to 4 min. for rats experiencing their first to sixth seizures. From the seventh seizure onwards, latency was prolonged to between 4 to 6 min. The first seizures were characterized by facial clonus, head nodding, or less commonly, forelimb clonus (seizure grades 1-3). In some subjects, forelimb clonus was lateralized to one side of the body before spreading to include the contralateral side. It was rare for rats to undergo signs of severe limbic seizures, grade 4 and 5, with the initial seizure induction. Rats whose first seizures were tonic-clonic usually died during the episode. Subsequent seizures tended to increase in both duration and severity. However, it was not unusual for a rat to have a lower grade seizure than the one induced previously. As seizures intensified in grade (4-5) to include body contractions, most rats fell onto their sides with tonic extension of all limbs. The frequency

of body contractions increased before gradually resolving. The rat continued to lie on its side with rapid respiration. Regaining of consciousness was marked by the animal righting itself and engaging in facial grooming with the forelimbs.

Analysis of data from seizure induction trials using linear regression indicates a high correlation between seizure length and seizure severity, ranked from 0 to 5, no seizures to severe tonic-clonic seizures, respectively (Figure 2 and Appendix II). This analysis revealed a general trend in the data: per-incremental increase in the seizure grade, 27 seconds is added to the length of the seizure. This value can be accepted with 95% certainty as the t-statistic value is greater than 2 (Appendix III); however, seizure duration varies with each trial and is not a constant for each seizure grade.

Linear regression analysis of the data for all rats that experienced hyperthermic seizures found a correlation between the number of seizures induced and the seizure duration (Figure 3 and Appendix III). Statistical analysis revealed a general trend in the data for this population: per-incremental increase in the number of seizures, an average of 22.8 seconds is added to the duration of the seizure. That is, seizures tended to increase in duration as the number of seizures increased. This can be accepted with greater than 95% confidence as the t-statistic value is 8.8 (Appendix IV).

Statistical analysis did not find a significant correlation between the number of seizures and the seizure grade. Thus, seizure duration tended to increase as the number of seizures

increased, but the subsequent seizures were not necessarily of higher grade. This conclusion is reflected in the recorded data.

Expression Pattern of BDNF mRNA

Among the normothermic control animals, the pattern and intensity of BDNF mRNA expression, as determined by densitometry, did not vary significantly with the number of exposures to 37 ^{0}C water. BDNF mRNA was highly localized to the pyramidal cell layer of hippocampal areas CA1 to CA3 and to the granule cell layer of the DG although it was detected in lesser amounts in the hilus, molecular cell layer, and stratum oriens and radiatum of CA1 and CA3 (Figure 4). The mean values for each of the regions examined are reported in Table 1. In the controls, the greatest amount of BDNF mRNA was detected in the granule cell layer of the DG, with a mean value 0.05415 $^+/_-$ 0.01 nCi/g.

In all seizure groups, autoradiograms showed BDNF mRNA expression was most highly localized to the pyramidal cell layer of hippocampal areas CA1 to CA3 and the granule cell layer of the DG. This localization can be seen in Figure 4, in which digitized images of the autoradiogram are converted to a color scale that has been calibrated to the densitometry values of coexposed ^{14}C standards. Figure 4 shows the areas where BDNF mRNA expression increased with the number of seizures experienced. The highest level of BDNF mRNA expression was observed in region #7, the granule cell layer of the DG. The granule cell layer demonstrated a greater than four-fold increase in BDNF mRNA in the 9 seizure group (mean value =0.22967 nCi/g) compared to the control (mean value =0.05415

nCi/g). All of the remaining regions--CA1 and CA3, including stratum oriens and stratum radiatum, the molecular layer of the DG and hilus--show only a 1.5 to 1.7-fold increase in expression after 9 seizures compared to the control. In particular, pyramidal cell layers of CA1 and CA3 show the least BDNF mRNA increase, only 1.5-fold (Table 1).

In looking at the set of data specific to each rat, regardless of the number of seizures induced, the greatest amount of BDNF mRNA expression, in descending order, was observed in region 7 (granule cell layer of the DG), then region 5 (CA3 pyramidal cell layer), region 2 (CA1 pyramidal cell layer), region 6 (hilus), region 8 (molecular layer), and region 4 (CA3 stratum radiatum). Stratum oriens and radiatum of CA1 had the smallest BDNF mean values of all the regions. Although the actual densitometry values varied between different animals, this pattern of differential BDNF mRNA expression (DG granular cell layer > CA3 pyramidal cell layer > CA1 pyramidal cell layer<hilus<molecular layer, etc.) was repeated in all of the subjects including the controls.

Statistical Analysis of Regional Differences in the Hippocampus

The Kruskal-Wallis one-way analysis for variance (K-W) demonstrated that there is a true difference in the densitometry values measured in the granule cell layer of the dentate gyrus (DG) among the control and seizure groups, and this can be accepted with greater than 98% certainty as the p value is less than .02. According to K-W, the difference in values measured in region 1 (stratum oriens of CA1) approached significance with a p value

between 0.10 and 0.05. Similarly, the differences in measured values for region 8, the molecular layer of the DG, approached significance with a p value slightly greater than 0.10 but less than 0.20. The remaining regions did not demonstrate a statistically significant difference between control and seizure groups. Appendix I shows the K-W results for all regions examined.

Inter-group comparisons of BDNF mRNA expression levels by the Mann-Whitney U test (M-W) are shown in Table 2. In region 7 (granular cell layer of the DG), the M-W test indicated a greater expression of BDNF mRNA in the 3 seizure group compared to the control group (the p value equals .028). Expression was greater in the 6 seizure group compared to the controls (the p value equals .028). There is greater expression in the 9 seizure group compared to the 3 seizure group (the p value equals .05) and to the control group (the p value equals .05). M-W analysis of the following groups did not yield a statistically significant difference in region 7: 1 seizure group versus control; 6 seizure group versus 3 seizure group; and 9 seizure group versus 6 seizure group. BDNF mRNA values obtained from the 3, 6, and 9 seizure groups were all greater than any values recorded in the 1 seizure group. However, there was not a sufficient number of subjects in the 1 seizure group (n=2) to produce a statistically significant difference using the M-W test (an n=3 was required for the p value to equal .05 and be statistically significant).

The M-W test was also used to compare measurements obtained in region 1 (stratum oriens of CA1) and region 8 (molecular layer of the DG). In both of these regions, the

expression of BDNF mRNA was found to be greater in the 9 seizure group than the control group. This can be accepted with greater than 95% certainty because the p value is .028 for both regions.

Discussion

I. BDNF mRNA Expression in the Rat

In the normothermic control rats, BDNF mRNA is most highly localized to the granule cell layer of the DG and pyramidal cell layer of CA1 to CA3. This is in agreement with prior published studies of BDNF mRNA expression in the normal adult rat (90, 91). Our study shows that, in the hippocampus, the granular cell layer of the DG has the highest density of hybridization to BDNF oligoprobes followed by the pyramidal cell layers. This contrasts with earlier studies by Ernfors et al. in which CA2 and CA3 demonstrated greater expression than the DG (90). The CA3 pyramidal cell layer is more densely labeled than CA1, and reports by Isackson et al. corroborate our finding (91). BDNF mRNA was also detected in lesser quantities in the hilus, the molecular layer of the DG, and stratum oriens of CA1 and stratum radiatum of CA1 and CA3.

II. BDNF mRNA Expression in Hyperthermic Seizures, Seizure Length, and Neuropathology

Previous investigations using the warm-water-immersion rat model of hyperthermic seizures have shown that short-duration seizures do not cause detectable neurodegeneration. In contrast, a seizure of prolonged duration, similar to status epilepticus, consistently produces a pattern of neurodegeneration with remarkable similarities to neuropathology observed in human TLE.

In the hippocampus, hyperthermic seizures of short-duration result in upregulation BDNF mRNA from control levels. The earliest

increase was detected after 3 seizures in the granular cell layer of the dentate gyrus, exhibiting a two-fold increase compared to controls. By 9 seizures, statistically significant increases in BDNF mRNA could be detected in stratum oriens of CA1, the hilus and granular cell layer of the DG compared to the control group. Peak elevation of BDNF mRNA was observed after 9 seizures in all hippocampal regions measured. This data demonstrates that the amount of BDNF mRNA expression is positively correlated with the number of seizures experienced. It also suggests that hyperthermic seizures can alter and enhance the hippocampal response to subsequent seizure activity, corroborating the conclusions made by Chen et al. that febrile seizures are not benign phenomena (92).

Given the known neuroprotective role of BDNF, upregulation of BDNF mRNA in the hippocampus during short-duration seizures, in which no neuron loss is detected, supports our hypothesis that BDNF protects cells from injury during short-duration hyperthermic seizures. Furthermore, regional differences in BDNF mRNA expression may offer an explanation for regional differences in hippocampal neuron survival after prolonged seizures (66). In the hyperthermia-induced status epilepticus rat, the dentate granular cell layer is relatively resistant to neuronal injury while the greatest amount of neurodegeneration is observed in CA1, and slightly less in CA3 (66). This pattern of neurodegeneration is similar to hippocampal neuron loss found in human TLE (29). In our short-duration seizure rats, the highest levels of BDNF mRNA are expressed in the dentate granular cell layer. In addition, the greatest average increase in BDNF mRNA, after recurrent short-

duration seizures, occurs in the dentate granular cell layer (4.24-fold), while all other hippocampal regions exhibited increases of 1.5- to 1.7-fold from control values. In comparison to the DG and CA3, the CA1 pyramidal cell layer exhibits the least BDNF mRNA expression. Furthermore, CA1 and CA3 pyramidal cell layers demonstrate the smallest BDNF mRNA response (1.5-fold increase compared to controls) to seizure activity of all the hippocampal regions examined. Collectively, these findings suggest that an insufficient BDNF response in vulnerable regions, may leave neurons susceptible to excitotoxic injury and death during prolonged seizure activity. We were unable to directly test this hypothesis because the status epilepticus rat generated in this experiment was used for a separate study of neurodegeneration, rendering it unsuitable for in situ hybridization. However, a recent study using the kainic acid (KA) rat model, in which status epilepticus is induced, correlated the induction of BDNF mRNA with cell survival in the hippocampus (93).

The increase in BDNF expression as a function of the number of seizures may contribute to the sprouting of granule cell recurrent collaterals into the inner molecular layer (IML) observed in this rat model. Prior studies describe a positive correlation between the number of hyperthermia-induced seizures in the rat and the extent of mossy fiber sprouting in the DG (66). It is possible to infer from our present data that a positive correlation also exists between the level of BDNF mRNA expression and the extent of mossy fiber sprouting in the DG. In vitro studies have demonstrated that exogenous BDNF can increase axonal branching of dentate granule

cells (82). It is plausible that the high levels of BDNF expression induced in the DG, compared to other hippocampal regions, is responsible for the unique sprouting of dentate granule cell "recurrent mossy fiber" axons.

One limitation of the present study is that we only examined BDNF mRNA levels one hour after seizure activity. Other studies employing different methods of seizure induction in the rat--KA injection, kindling, hilar lesioning (HL)--report that the magnitude and expression pattern of this mRNA changes depending on the amount of time elapsed after the seizure. Peak levels of BDNF mRNA are reported in the DG soon after seizure activity; whereas, peak levels in CA1 and CA3 occur hours later (94). Nonetheless, all of these studies agree with our observation that the DG is the first region to show upregulation of BDNF mRNA after seizure activity (91, 94, 95). Similarly, in other rat models, the maximum increase in seizure-induced BDNF mRNA expression occurs in the DG. This increase is two- to six-times the peak response in CA1 or CA3 (91, 94, 95).

Statistical analysis of our data reveals a positive correlation between the number of hyperthermic seizures induced and the seizure duration. Linear regression demonstrates that 22.8 seconds is added to the seizure duration per increase in the number of seizures ($p<0.05$). It is possible that, as the number of recurrent seizures is increased, the progressively augmented BDNF response enhances neuronal excitability which lengthens the seizure duration. In fact, BDNF has been shown, in rat models, to enhance excitatory synaptic transmission in vivo and in vitro (83-86). In vitro studies

show that BDNF can enhance excitatory synaptic transmission in the DG of human TLE patients (89).

The results of our study suggest a multi-functional role for BDNF upregulation during hyperthermic seizures. It is possible that BDNF induction, in high levels and immediately following seizure activity, protects neurons from excitotoxic injury and regulates mossy fiber sprouting. In neurons expressing BDNF in lower amounts and later in time after seizure activity, such as pyramidal cells of CA1 and CA3, BDNF functions primarily to enhance neuronal excitability. These different functions of BDNF may be accomplished if different signaling cascades are coupled to the TrkB receptor. Which function is activated may depend either on timing or the extent of BDNF induction or on different signaling cascades which are unique to specific cell populations. Although elucidation of the precise mechanisms which mediate the actions of BDNF in hyperthermic seizures is beyond the scope of the present study, our data does suggest a multi-functional and more complex role for BDNF in hyperthermic seizures than previously proposed.

III. Relevance for Human TLE and Febrile Convulsions

The precise role of FCs in the etiology of human TLE remains to be determined. Whether or not FCs contribute to the formation of the patterns of hippocampal neuron loss sometimes associated with human TLE--MTS and hippocampal sclerosis--is still a topic of debate among epilepsy researchers. The issue is a long-standing and controversial one, and there is data in the literature that argues for and against a correlation between a history of FCs and

the subsequent development of TLE. If FCs contribute to the development of TLE in patients, then early recognition and treatment of FCs is crucial for preventing the neurological sequelae. Our findings in the rat model of hyperthermic seizures may offer valuable insight into the effects of humans FCs and its role in the pathogenesis of TLE.

Remarkable similarities exist between the pattern of BDNF mRNA expression in the hippocampus of the rat model of hyperthermic seizures and human TLE. Human, non-seizure autopsy cases demonstrate that, in the hippocampus, BDNF mRNA is mainly localized to the DG granule cell layer and stratum pyramidale of CA3. Low levels of this mRNA were detected in the hilus and, sporadically, in CA1. These regional differences in BDNF mRNA expression are also maintained in the hippocampus of the adult rat.

Furthermore, hyperthermic seizures in the rat induce increases in BDNF mRNA in the hippocampus. In tissue resected from human TLE patients, there is an 89% increase in BDNF mRNA in the DG granule cell layer compared to autopsy controls. Lesser increases were seen in the hippocampal fields. However, in situ hybridization showed more intense BDNF mRNA labeling in the CA3 pyramidal cells in TLE compared to autopsy controls. BDNF mRNA could not be detected in the CA1 region of TLE patients due to extensive hippocampal sclerosis (88). These differences in the regional upregulation of BDNF mRNA expression in human TLE are also observed in the hippocampus of the hyperthermic seizure rat.

In addition, our previous studies have shown that hyperthermic seizures, which are prolonged in duration, produce a pattern of hippocampal neuron loss that bears marked resemblance to MTS pathology found in TLE: 1) The granule cells of the DG are resistant to injury, 2) CA3 pyramidal cell layer demonstrates neuron loss, 3) the greatest neuron losses are detected in the CA1 pyramidal cell layer (66). By comparison, hyperthermic seizures of short-duration do not produce detectable neuron loss in the rat. These findings corroborate published human data which report that the severity of hippocampal neuron loss in TLE is strongly associated with prolonged FCs (51, 55, 56).

Thus, in both the hyperthermic seizure rat and human TLE patients, the degree of BDNF mRNA expression in the hippocampus appears to associated with the survival of hippocampal neurons. Based on these similarities, it is possible to infer from the rat model of hyperthermic seizures that, in humans, FCs induce an upregulation of BDNF mRNA expression in the hippocampus. The increase in BDNF mRNA confers neuroprotective properties against seizure-induced neuronal injury and death. However, during prolonged FCs, there is an inadequate BDNF mRNA response in certain regions in the hippocampus, making these neuron populations more susceptible to excitotoxic injury. In this way, prolonged FCs can produce the MTS that leads to the development of human TLE. In addition to neuroprotection, the increase in BDNF mRNA in human TLE may increase excitability of some hippocampal neurons, further contributing to epileptogenicity.

Although more research is required to confirm this hypothesis in human TLE patients, our study using the rat model suggests one possible mechanism through which FCs could act to produce MTS and TLE.

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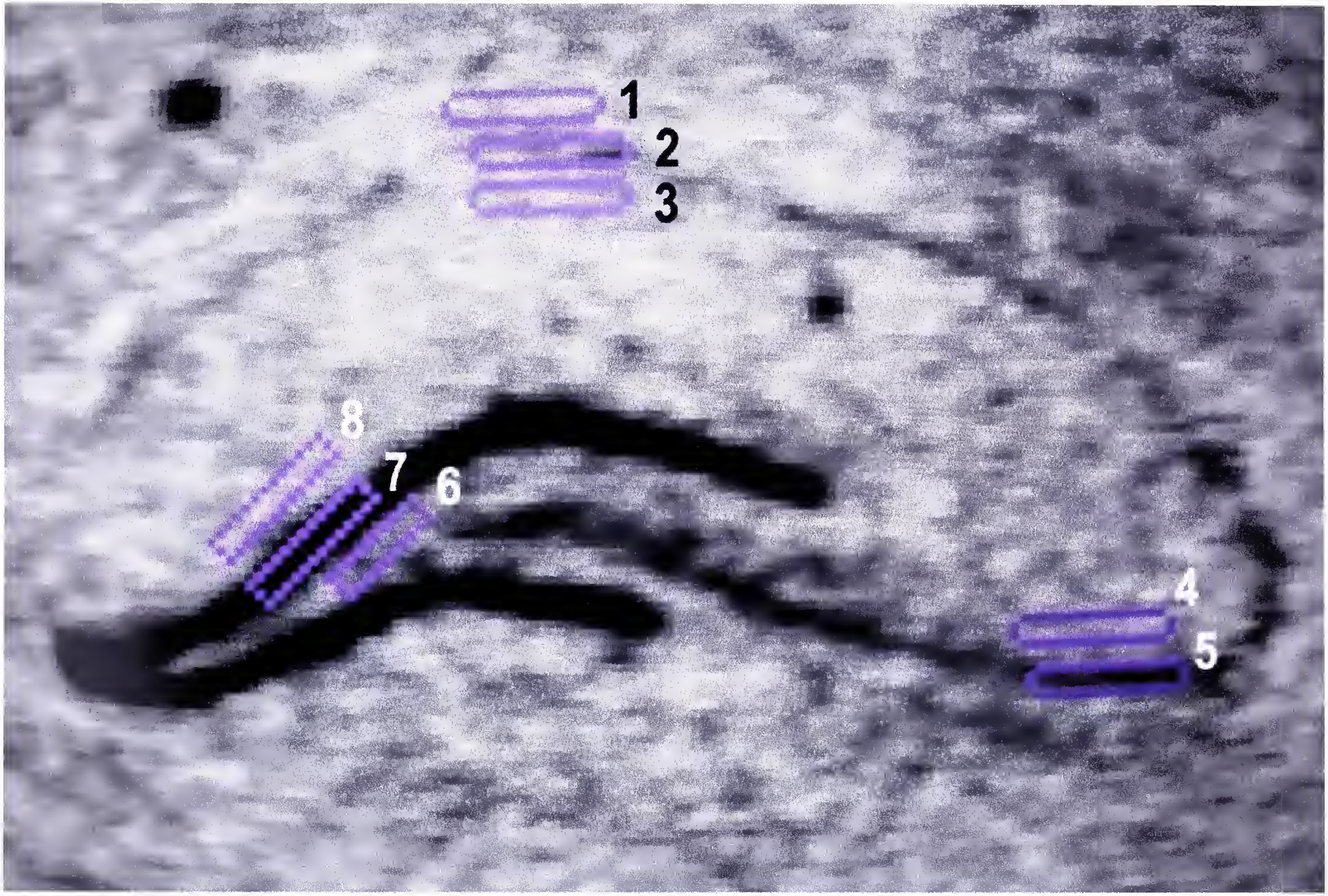

Figure 1.

Digitized image of autoradiogram showing localization of BDNF mRNA oligonucleotide probe in the hippocampus. Colored boxes outline regions for which densitometry values were obtained. The numbers correspond to the following hippocampal anatomy: (1) CA1 stratum oriens, (2) CA1 pyramidal cell layer, (3) CA1 stratum radiatum, (4) CA3 stratum radiatum, (5) CA3 pyramidal cell layer, (6) hilus, (7) granular cell layer of the dentate gyrus, and (8) molecular layer.

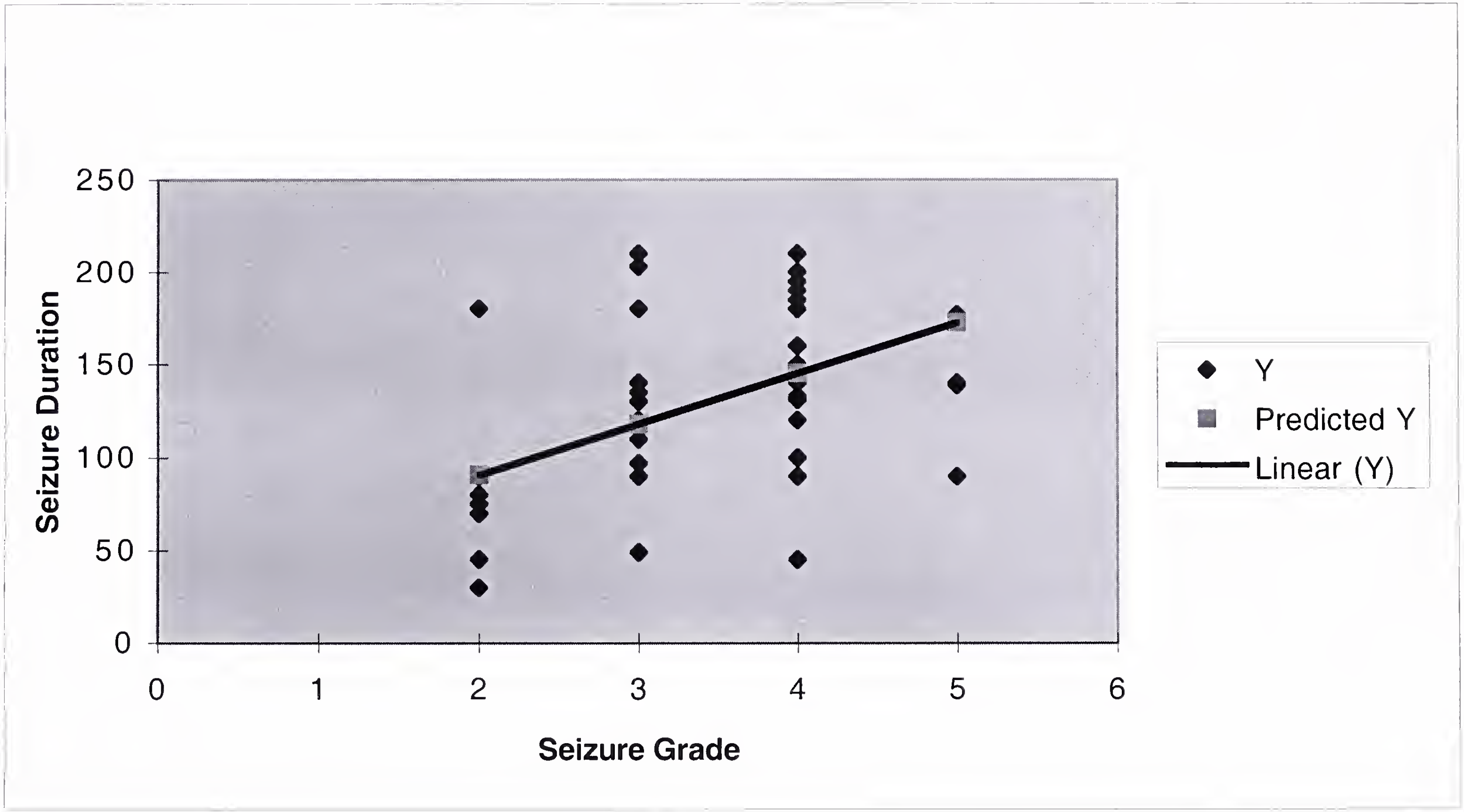

Figure 2. Graph of linear regression showing correlation between seizure grade and duration. Per-incremental increase in seizure type, 27 secs. is added to the seizure duration. This can be accepted with 95% certainty (t-stat >2).

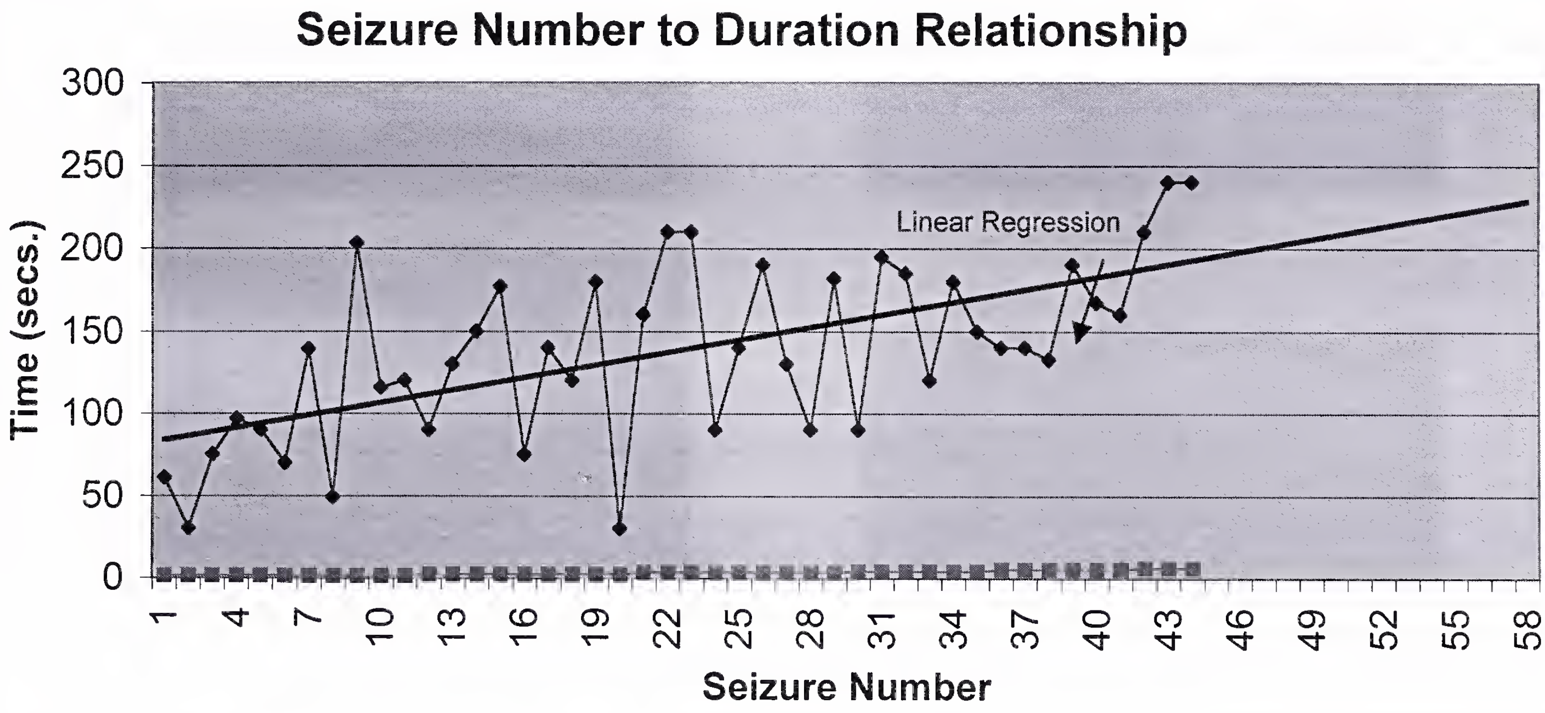

Figure 3. Graph of linear regression showing strong correlation between seizure duration and the number of seizures. This correlation can be accepted with >95% certainty (t-stat equals 8.8).

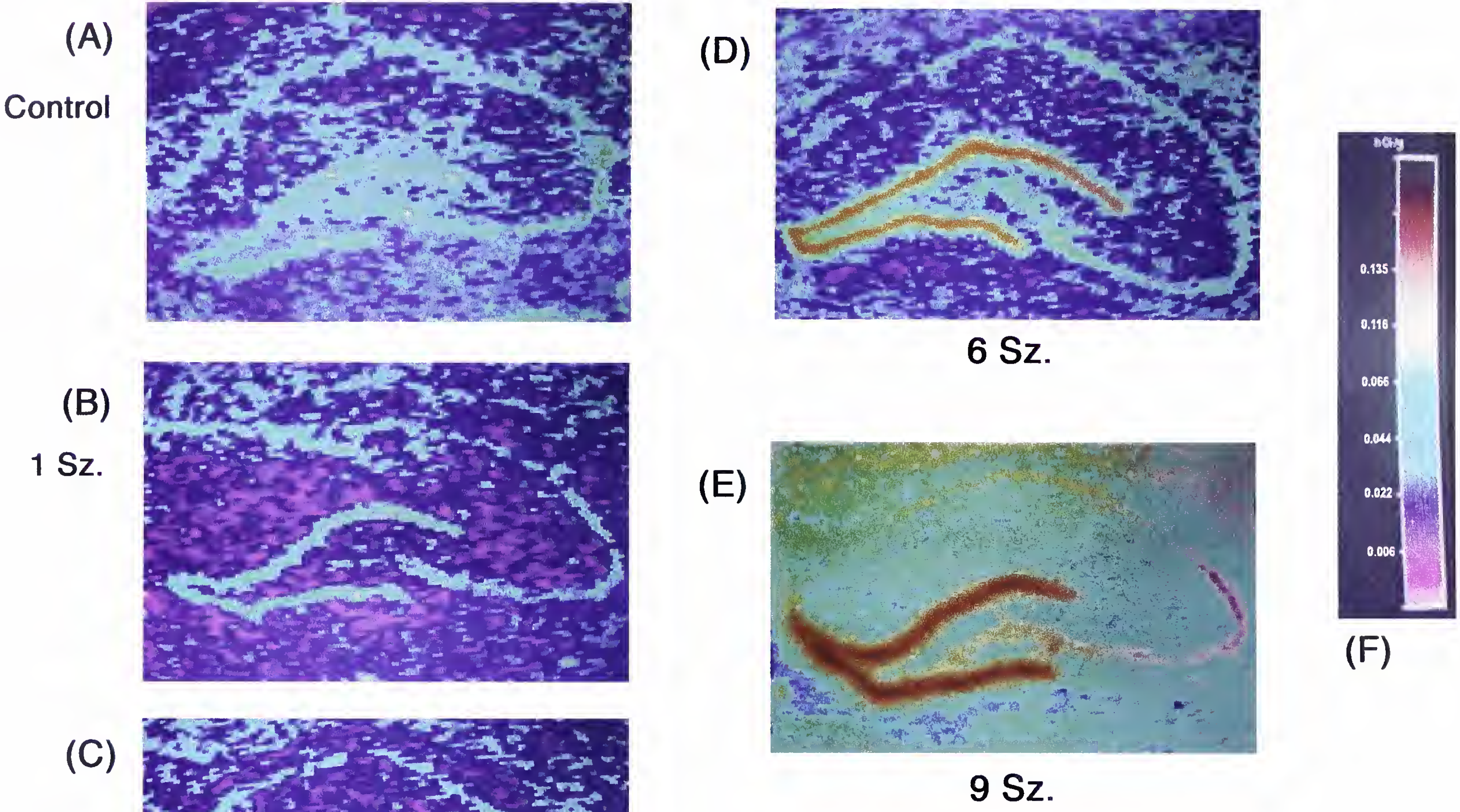

Figure 4. Digitized densitometry images of the rat hippocampus and dentate gyrus with calibrated color scale enhancement to show differential expression and localization of BDNF mRNA in these structures as a function of the number of hyperthermia-induced seizures experienced. Note the marked increase in BDNF mRNA in the hippocampus and dentate gyrus between the control and 9 seizure rats. (A) Control rat, (B) 1 seizure, (C) 3 seizures, (D) 6 seizures and (E) 9 seizures. (F) Color scale calibrated to densitometry values of coexposed C-14 standards expressed in units of nCi/g.

	CA1 Stratum Oriens	CA1 Pyramidal Cell Layer	CA1 Stratum Radiatum	CA3 Stratum Radiatum	CA3 Pyramidal Cell Layer	Hilus	DG Granular Cell Layer	DG Molecular Cell Layer
1 Sz. group								
Average	0.0246	0.03505	0.02395	0.0248	0.054	0.03375	0.0847	0.0272
St.Deviation	0.00650538	0.00714178	0.00487904	0.0066468	0.01697056	0.01096016	0.0337997	0.00636396
3 Sz. group								
Average	0.02007	0.03383	0.02077	0.02257	0.04883	0.0316	0.1176	0.0287
St.Deviation	0.00325013	0.00427356	0.00346458	0.00343851	0.00450592	0.00185203	0.01134592	0.00498698
6 Sz. group								
Average	0.02583	0.03773	0.0259	0.02867	0.048	0.0337	0.1516	0.03313
St.Deviation	0.00076376	0.00283078	0.0008544	0.0011547	0.00458258	0.00467654	0.02130446	0.00458839
9 Sz. group								
Average	0.03833	0.05567	0.04	0.04267	0.072	0.05033	0.2297	0.045
St.Deviation	0.01159023	0.01650253	0.01452584	0.01379613	0.0226495	0.0170098	0.10303559	0.014
CONTROLS								
Average	0.02278	0.03638	0.02365	0.0257	0.04603	0.02935	0.05415	0.026
St.Deviation	0.00238939	0.00338465	0.00367922	0.00531789	0.00372592	0.00306105	0.01093938	0.00335062
ANOVA	$0.05<p<0.10$	$0.30<p<0.20$	$0.10<p<0.20$	$0.20<p<0.30$	$0.30<p<0.50$	$0.10<p<0.20$	$0.01<p<0.02$	$0.20<p<0.10$

Table 1. Mean densitometry values for the hippocampus and Kruskal-Wallis Anova Results

	CA1 S. Oriens	DG Granular Cell Layer	DG Molecular Layer
1sz vs. Control		p=0.133	
3sz vs. Control		**p=0.028**	
3sz vs. 1sz		p=0.10	
6sz vs. control		**p=0.028**	
6sz vs. 1sz		p=0.10	
6sz vs. 3sz		p=0.10	
9sz vs. Control	**p=0.028**	**p=0.028**	**p=0.028**
9sz vs. 1sz		p=0.10	
9sz vs. 3sz		**p=0.05**	
9sz vs. 6sz		p=0.20	

*** P values in bold-face type are accepted with greater than 95% certainty as $p \leq 0.05$.**

Table 2. Comparison of BDNF mRNA expression between groups with the Mann-Whitney U test.

	#1	#2	#3	#4	#5	#6	#7	#8
1 Sz. group								
9B1H	0.02	0.03	0.0205	0.0201	0.042	0.026	0.0608	0.0227
4B1H	0.0292	0.0401	0.0274	0.0295	0.066	0.0415	0.1086	0.0317
3 Sz. group								
5A3H	0.0233	0.0364	0.0223	0.0244	0.0491	0.0323	0.1109	0.028
4A3H	0.0168	0.0289	0.0168	0.0186	0.0442	0.0295	0.1112	0.0241
3A3H	0.0201	0.0362	0.0232	0.0247	0.0532	0.033	0.1307	0.034
6 Sz. group								
15A6H	0.0265	0.0362	0.0267	0.03	0.053	0.0391	0.1288	0.0384
10A6H	0.026	0.041	0.025	0.028	0.044	0.031	0.171	0.03
8A6H	0.025	0.036	0.026	0.028	0.047	0.031	0.155	0.031
9 Sz. group								
14A9H	0.049	0.072	0.054	0.053	0.093	0.067	0.344	0.059
12A9H	0.04	0.056	0.041	0.048	0.075	0.051	0.201	0.045
6A9H	0.026	0.039	0.025	0.027	0.048	0.033	0.144	0.031
CONTROLS								
3B1C	0.0228	0.0377	0.0229	0.0226	0.0499	0.0288	0.0677	0.0262
18A3C	0.0203	0.0358	0.0207	0.0202	0.0472	0.0256	0.0419	0.0218
17A6C	0.022	0.032	0.022	0.028	0.041	0.03	0.05	0.026
20A9C	0.026	0.04	0.029	0.032	0.046	0.033	0.057	0.03
KRUSKAL-WALLACE	**H=7.86**	**H=5.38**	**H=6.12**	**H=5.78**	**H=3.574**	**H=6.025**	**H=12.36**	**H=7.13**

The column number corresponds to the following hippocampal regions: 1) CA1 stratum oriens, 2) CA1 pyramidal cell layer, 3) CA1 stratum oriens, 4) CA3 stratum radiatum, 5) CA3 pyramidal cell layer, 6) hilus, 7) dentate granular cell layer and 8) DG molecular layer.

APPENDIX I. Densitometry values for hippocampal regions and results of Kruskal-Wallis analysis.

	Sz # 1	#2	#3	#4	#5
9B1H	61 sec/ G2				
4B1H	30sec/ G2				
5A3H	75sec/ G2	90sec/ G3	160sec/ G4		
4A3H	97sec/ G3	130sec/ G3	210sec/ G4		
3A3H	90sec/ G3	150sec/ G4	210sec/ G3		
15A6H	70sec/ G2	177sec/ G5	90sec/ G3	90sec/ G3	140sec/ G3
10A6H	139sec/ G5	75sec/ G2	140sec/ G4	195sec/ G4	140sec/ G3
8A6H	49sec/ G3	140sec/ G5	190sec/ G4	185sec/ G4	133sec/ G3
14A9H	203sec/ G3	120sec/ G3	130sec/ G3	120sec/ G4	190sec/ G4
12A9H	116sec/ G3	180sec/ G4	90sec/ G3	180sec/ G3	167sec/ G5
6A9H	120sec/ G3	30sec/ G2	182sec/ G4	150sec/ G3	160sec/ G3

Appendix II. Rat Seizure Duration and Seizure Grade (G 1-5) Data

	#6	#7	#8	#9
15A6H	210sec/ G4			
10A6H	240sec/ G4			
8A6H	240sec/ G4			
14A9H	170sec/ G4	192sec/ G3	160sec/ G5	283sec/ G3
12A9H	238sec/ G4	200sec/ G4	270sec/ G4	320sec/ G4
6A9H	117sec/ G3	256sec/ G4	330sec/ G5	356sec/ G5

Appendix II. (Continued)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.46683308
R Square	**0.21793313**
Adjusted R Squ	0.20259848
Standard Error	44.0903111
Observations	53

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	27627.1359	27627.1359	14.2118148	0.00042549
Residual	51	99141.732	1943.95553		
Total	52	126768.868			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	36.3959391	24.6347187	1.47742459	0.14571103	-13.0602843	85.8521625	-13.0602843	85.8521625
X Variable 1	**27.2629442**	7.23182627	**3.76985607**	0.00042549	12.7444583	41.78143	12.7444583	41.78143

* Seizure grade explains up to 22% of the length of the seizure.

Comment: Very high given length of seizure is the result of many factors.
Seizure type just one variable explaining so much of the length of seizure

* Per-incremental increase in the seizure grade, 27 seconds is added to the duration of the seizure

This value can be accepted with 95% certainty as the t-statistic is greater than 2.

Appendix III. Linear regression data showing strong correlation between seizure grade and seizure duration

SUMMARY OUTPUT

Model: Ordinary Least Squares Regression
Duraction$_t$ = intercept + beta*Seizure$_t$ + error$_t$

Regression Statistics	
Multiple R	0.765988
R Square	0.586737
Adjusted R	**58%**
Standard E	46.77453
Observatio	56

ANOVA

	df	*SS*	*MS*	*F*	*ignificance F*
Regression	1	167737.2	167737.2	76.66736	6.06E-12
Residual	54	118144.2	2187.856		
Total	55	285881.4			

	Coefficients	*andard Errc*	***t Stat***	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	71.62282	11.89975	6.018849	1.59E-07	47.76523	95.48041	47.76523	95.48041
Seizure #	**22.8**	2.601167	**8.8**	6.06E-12	17.56076	27.99082	17.56076	27.99082

1) The truncated nature of the independent variable (the number of seizures cannot be negative) and the potential for positive serial correlation in the independent variable will likely bias the results downward. As such the true relationship may be understated.

2) Under a generalized setting, it is safe to say that a clear statistical relationship exists between the number of seizures and the duration of the seizure.

3) Results indicate that the number of seizures can explain the duration of the seizure 58% of the time.

4) The coefficient of the number of seizures (22.8) suggests that increasing the number of seizures by just one will add an additional 22.8 seconds to the duration of the seizure.

5) The associated t-stat shows that the coefficient of the number of seizures is very statistically significant - well within the 95% confidence interval level.

Appendix IV. Linear regression data showing correlation between the number of seizures and seizure duration.

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